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Reduced Biocide Susceptibility in *Candida albicans* Biofilms▽

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***Candida* biofilm formation is common during infection and environmental growth. We tested the impacts of three biocides (ethanol [EtOH], H_2O_2, and sodium dodecyl sulfate) on *Candida albicans*, *C. parapsilosis*, and *C. glabrata* biofilms. Higher concentrations of the biocides were required for efficacy against biofilms than for efficacy against planktonic controls. A combination study with two biocides (EtOH and H_2O_2) and fluconazole demonstrated that the combination had enhanced efficacy.**

Candida species cause a wide spectrum of diseases, including hospital-acquired and device-associated infections (33). In the hospital setting, *Candida* persists on colonized individuals and medical equipment (10). When it is growing on a surface, such as a medical device, *Candida* adapts to a biofilm lifestyle (14, 15, 19, 22). Biofilm formation is a common mode of growth during infection and survival in the environment (11, 15, 19, 22, 34). Biofilms consist of cells attached to a surface and embedded in a matrix produced by the organisms (13). Phenotypic changes are associated with biofilm formation, and among these, resistance to antifungal agents has been implicated in the difficulty of treatment of biofilm infections (2, 5, 21, 27, 36–38). In fact, removal of the *Candida*-infected medical device is nearly always required for cure of the infection (33). Biofilm resistance to antifungals has been well described (3, 4, 8, 16, 20, 37). Comparisons of biofilm cells and planktonic cells, which are the free-floating counterparts of biofilm cells, demonstrate that biofilms have up to a 1,000-fold increased resistance (8, 17, 25, 32). *Candida* biofilm susceptibility to biocides has received less attention. Biocides are chemical or physical agents that inactivate microorganisms. Because they commonly demonstrate a broad spectrum of activity, the agents are often used as topical therapies for patients or environmental disinfectants (12, 26, 41). Several studies of *C. albicans* biofilms have examined the activities of chlorhexidine, ethanol (EtOH), hydrogen peroxide, betadine, and sodium hypochlorite, although the findings have not been consistent (7, 9, 23, 42, 43, 45, 46). These investigations have not routinely included comparisons of biofilms with planktonic cell cultures. Also, experimental designs have not accounted for the difference in cell numbers between planktonic cell cultures and intact biofilms.

The purpose of this study was (i) to compare the activities of different biocides against *Candida albicans*, *C. parapsilosis*, and *C. glabrata* biofilm and planktonic cells and (ii) to investigate the impacts of biocides on the activity of an antifungal, fluconazole, for the prevention and treatment of *Candida* biofilms (*C. albicans* only). We chose to study EtOH, H_2O_2, and sodium dodecyl sulfate (SDS) because they are relatively commonly used and the have different modes of action (26). Fluconazole was selected for use in the combination therapy investigations due to the resistance of *Candida* biofilms to this common antifungal (3, 9, 27, 39).

C. albicans (strains DAY 185 and K1), *C. parapsilosis* (strain 5986), and *C. glabrata* (strain 5740) biofilms were grown in 96-well polystyrene plates as described previously (30, 37). The wells of the plates were inoculated with either a standard CLSI (formerly NCCLS) inoculum or a higher inoculum in RPMI-morpholinepropanesulfonic acid on the basis of the burden of viable cells in the biofilm assay at the start of therapy (10^6 to 10^7 cells/well). After 24 h of incubation at 30°C, the biofilms were washed twice with phosphate-buffered saline. For the prevention assays, the biocides were added at the time of inoculation. For the treatment assays, the biocides and fresh medium were added to mature biofilms after 24 h of biofilm growth. Dilutions of the biocides were studied to include concentrations generally effective against planktonic organisms (26). The concentration ranges studied were as follows; H_2O_2, 1 to 1,000 mM; EtOH, 0.05 to 50%; and SDS, 0.0004 to 0.4%. After a 24-h incubation at 30°C, a 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2*H*-tetrazolium hydroxide reduction assay was performed (30, 37).

We determined the drug concentrations associated with a 50% reduction (50% effective concentration [EC_{50}]) and an 80% reduction (EC_{80}) in the optical density compared to that for the no-drug controls. For the studies with planktonic cells, the MICs of the biocides were measured by using CLSI endpoints. To account for the increased number of cells in the biofilm (10^6 to 10^7 CFU/well) compared to the number recommended for use in the CLSI method (0.5×10^3 to 2.5×10^3 CFU/ml), planktonic cell MICs were adjusted to a similar inoculum (18, 29). The assays were performed in triplicate on two occasions.

The impacts of the biocides in combination with fluconazole (concentration range, 0.0625 to 1,000 μg/ml) on the treatment of mature biofilms were similarly examined by using a checkerboard format. Mature biofilms (24 h) were incubated in the presence of the biocide and antifungal combination for 24 h, and the endpoints were assessed as described above. Fractional inhibitory concentration (FIC) indices were used to estimate

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TABLE 1. Effects of biocides on *Candida* biofilm and planktonic cells[a]

Biocide	Strain	Plank. cell MIC	Modified plank. cells[b]		Biofilm				Fluc FIC[c]
					Treatment		Prevention		
			EC_{50}	EC_{80}	EC_{50}	EC_{80}	EC_{50}	EC_{80}	
EtOH	*C. albicans* DAY 185	3	6	13	35	40	35	35	<0.5
	C. albicans K1	1.5	6	13	25	25	NA	NA	NA
	C. parapsilosis 5986	1.5	6	6	25	25	NA	NA	NA
	C. glabrata 5740	3	13	13	25	25	NA	NA	NA
H_2O_2	*C. albicans* DAY 185	16	31	63	175	200	20	40	<0.27
	C. albicans K1	1	63	63	250	250	NA	NA	NA
	C. parapsilosis 5986	1	63	63	125	250	NA	NA	NA
	C. glabrata 5740	16	16	31	125	500	NA	NA	NA
SDS	*C. albicans* DAY 185	0.01	0.05	0.05	0.15	0.15	0.05	0.1	1
	C. albicans K1	0.01	0.03	0.05	0.05	0.1	NA	NA	NA
	C. parapsilosis 5986	0.01	0.03	0.05	0.05	0.1	NA	NA	NA
	C. glabrata 5740	0.01	0.03	0.03	0.05	0.05	NA	NA	NA

[a] Plank., planktonic; Fluc, fluconazole; NA, not applicable. The units for the MICs, EC_{50}s, and EC_{80}s are percent for EtOH and SDS and mM for H_2O_2.
[b] The inoculum used for modified planktonic cell EC_{50} and EC_{80} testing was identical to the cell density used for the mature biofilms (10^6 to 10^7 cells/well). The endpoint reading was based on the optical density at 550 nm.
[c] FIC is equal to [(EC_{50} of drug A in combination)/(EC_{50} of drug A alone)] + [(EC_{50} of drug B in combination)/(EC_{50} of drug B alone)]; values less than 0.5 indicate an enhanced interaction.

the impact of each biocide on the activity of fluconazole, as described previously (30, 44).

EtOH, H_2O_2, and SDS were effective at reducing the metabolic activities of the *C. albicans* biofilms at concentrations commonly used for disinfection (26). However, the concentrations of the biocides required to inhibit growth were higher for biofilms than for planktonic cell cultures containing similar numbers of cells (Table 1). The concentrations needed to decrease the burden of mature biofilm cells by 50% were from 2- to 10-fold higher for biofilm cell inhibition than for planktonic cell inhibition, as follows: for EtOH, 25 to 35%; for H_2O_2, 125 to 250 mM; and for SDS, 0.05 to 0.15%. Decreasing the biofilm burden by 80% required even higher concentrations of EtOH and H_2O_2 (Table 1) (all P values were <0.05). Similarly high concentrations of EtOH and SDS were needed to prevent *C. albicans* biofilm formation. However, lower concentrations of H_2O_2 (40 mM) prevented biofilm formation (Table 1). Two of the biocides, EtOH and H_2O_2, potentiated the activity of fluconazole against *C. albicans* biofilms. However, SDS did not enhance the action of fluconazole. The positive impacts of these cell wall-perturbing agents on the activity of fluconazole along with the changes in the *Candida* cell wall during biofilm growth suggest a potential role for cell wall integrity in biofilm resistance (24, 30).

These disinfectants were able to affect *Candida* cell viability. However, the concentrations of biocides required for efficacy against biofilm cells were greater than those associated with the killing of planktonic cells, suggesting that a reduction in susceptibility is associated with biofilms. These data suggest that concentrations higher than those previously thought necessary may be needed to disinfect contaminated medical devices and equipment. Further studies with biofilm models may be useful to determine the biocide concentrations necessary for disinfection and biofilm eradication.

EtOH and H_2O_2 enhancement of the activity of fluconazole may prove to be useful in the treatment and disinfection of *Candida* biofilms associated with medical devices and equipment. H_2O_2 has been used for disinfection of oral hygiene devices and contact lenses (6, 28, 40). Also, the utility of ethanol (25 to 70%) as central venous catheter lock therapy is under investigation (1, 31, 35). The current study suggests that azole drugs, such as fluconazole, may act to enhance the activities of these biocides when they are used to prevent or treat fungal biofilms.

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